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Effect of relaxin on semen quality parameters of cryopreserved stallion semen

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Abstract

The aim of the study was to test the effect of different concentrations of relaxin, added in the extender medium during pre-freezing incubation time, on sperm quality parameters of equine frozen-thawed spermatozoa. Semen samples, collected from 3 proven fertility stallions, were filtered, diluted with BotuSemen® and centrifuged at 600 *g* for 10 min. The sperm pellets were resuspended in freezing medium BotuCrio® to a final concentration of $50x10^6$ sperm/ml. The diluted semen was divided into 5 experimental groups supplemented with 0 (control), 12.5, 25, 50 and 100 ng/ml of relaxin. Semen samples were packed in 0.5 ml straws, equilibrated at 5°C for 30 min, exposed to vapor of liquid nitrogen (LN_2) for 15 min and plunged into LN_2. After thawing, semen samples were evaluated for motility and velocity parameters, sperm vitality, mitochondrial membrane potential, apoptosis in addition to plasma membrane and DNA integrities. Sperm motility parameters and the percentage of viable spermatozoa were significantly improved in relaxin-treated samples immediately after thawing and after 30, 60, 90 and 120 min. of incubation, with highest values recorded when 12.5 and 25 ng/ml relaxin were used. Moreover, relaxin, at all tested concentrations, significantly improved the sperm mitochondrial membrane potential and decreased the percentage of apoptotic cells compared to the control group. Plasma membrane and DNA integrities were not affected by relaxin addition. In conclusion, the supplementation of relaxin in the extender before semen cryopreservation, especially at 12.5 and 25 ng/ml, has a positive stimulatory effect on semen quality parameters of frozen-thawed stallion semen.

Keywords; Stallion semen; Cryopreservation; Relaxin; Sperm quality

1. Introduction

Artificial insemination, with fresh, cooled, or frozen semen, is one of the assisted reproductive techniques commonly used in global equine industry (Freitas et al., 2016). Therefore, semen quality, intended as the ability to accomplish fertilization, is the most important factor for successful horse breeding programs (Magistrini et al., 1996; Parlevliet and Colenbrander, 1999; Gadella et al., 1999; Stradaioli, 2004). The advances in stallion semen cryopreservation resulted in an improvement of equine breeding industry by allowing the worldwide distribution of superior genetic resources avoiding the risks

associated with transportation and natural mating (Neild et al., 2003; Miller, 2008; Arruda de Oliveira et al., 2013). Despite all the precautions, the fertility of frozen thawed stallion semen remains low compared to fresh or cooled semen (Gibb and Aitken, 2016). Many of the deleterious effects induced by cryopreservation may be attributed to the osmotic stress induced by the ice crystals formation (Gibb and Aitken, 2016). Frozen-thawed spermatozoa have demonstrated various degree of damage such as reduced viability and motility as well as perturbations in membrane integrity with consequent loss of sperm fertilizing ability or even sperm death (Arruda de Oliveira et al., 2013; Watson, 2000; Uysal and Bucak, 2007). Furthermore, stallion spermatozoa contain high level of polyunsaturated fatty acids making these cells highly susceptible to reactive oxygen species (ROS) therefore inducing membrane lipid peroxidation (Neild et al., 2003; García et al., 2011; Gibb et al., 2013). In order to improve the quality of frozen-thawed semen, researchers attempts to refine the extender compositions by testing new additives to improve the sperm activity, the plasma membrane integrity and sperm fertility (Arruda de Oliveira et al., 2013; Ghallab et al., 2017).

Relaxin, an insulin superfamily regulatory peptide, has been identified in boar testes (Kohsaka et al., 2009) and in human seminal plasma (Ferlin et al., 2012). It has been suggested to have a physiological influence on sperm motility and fertility via specific cell-surface receptors on spermatozoa (Kohsaka et al., 2003). Relaxin has been demonstrated to improve motility of human (Ferlin et al., 2012), bovine (Miah et al., 2007) and porcine (Miah et al., 2008; Feugang et al., 2015) spermatozoa. In addition, relaxin has been found to induce capacitation and acrosome reaction in fresh and frozen-thawed porcine (Miah et al., 2008) and bovine semen (Miah et al., 2011). Furthermore, relaxin improved the fertilizing ability of porcine (Han et al., 2006) and buffalo spermatozoa (Elkhawagah et al., 2013; Elkhawagah et al., 2015). However, to the best of our knowledge, no studies have been published to date on the effects of relaxin on quality parameters of equine spermatozoa. In previous studies, conducted in other species, the effect of relaxin supplementation in the sperm-thawing media has been investigated at the end of the cryopreservation process (Miah et al., 2008; Miah et al., 2011; Elkhawagah et al., 2013; Elkhawagah et al., 2015). In the present study, a different methodological approach was used, indeed different concentrations of relaxin were added before sperm cryopreservation procedures and different fertility parameters were investigated. This methodological approach may be more suitable for in field applications, avoiding any type of treatment of the semen after thawing procedures and/or before the artificial insemination.

2. Material and methods

2.1. Semen collection and dilution

Three commercial proven fertility stallions (10- to 13-year-old ages) used for commercial purpose, were enrolled in this study. They were in good general condition without reporting any current or past health problems. Horses were housed in the same stud (Vigone, Turin, Italy) and were managed similarly for feeding and activity. Physical examination of the genitalia by palpation and ultrasonography identified no abnormalities. The stallions showed good libido. Semen samples were obtained once per week for 6

consecutive weeks towards the end of the breeding season when the commercial request was reduced. In detail, a total of 18 ejaculates (6 ejaculates for each stallion) were collected using artificial vagina (Colorado model Equine Artificial Vagina; ARS, Chino-CA, USA) pre-warmed at 45-50 °C. Semen samples were collected in a plastic bottle and filtered immediately after collection to separate gel fraction. Sperm motility, concentration, viability and sperm morphology were evaluated. Samples were diluted using double amount of skimmed milk and kept in water bath at 24 °C for 10 min then centrifuged at 600 *g* for 10 min. After centrifugation, supernatant was removed and the sperm pellets were resuspended using freezing medium (BotuCrio®, Botupharma, USA) to a final concentration of 50 x 10^6 sperm/ml. The diluted semen samples from the three stallions were always pooled together. This procedure was aimed to eliminate individual stallion variability (Seifi-Jamadi et al., 2016; Shojaeian et al., 2018; Nouri et al., 2018). Samples were divided into 5 experimental groups and supplemented with relaxin (SRP3147, Sigma-Aldrich, Italy) at different concentrations: 0 (control), 12.5, 25, 50 and 100 ng/ml. Semen of different experimental groups was packed in 0.5 ml polyvinyl straws (IMV, France) and kept in refrigerator at 5 °C for 30 min for equilibration. Then, straws were placed 4 cm over liquid nitrogen (LN_2) vapor for 15 min with an approximate temperature of vapor of 130 °C and then directly plunged into LN_2 for storage (Cristanelli et al. 1985). After one week, frozen straws were thawed in water bath at 37 °C for 60 s for subsequent procedures.

2.2. Assessment of sperm motility and velocity parameters

After thawing, semen was incubated at 37 °C and motility and velocity parameters were evaluated at 0, 30 min, 60 min, 90 min and 120 min of incubation using the Computer Assisted Sperm Analyzer (CASA; Hamilton Thorne, Inc., Beverly, MA, USA) by using a 10x objective at 37 °C and the SETUP specific for the equine species. In detail, the parameters were set as follows: 40 frames acquired at 60 frames/s; minimum contrast 80; minimum cell size 5 pixels; trajectory speed cutoff 20 µm/s; progressive motility cutoff 50 µm/s and linear motility 60%; linear motility cutoff 0 µm/s. CASA analyses were conducted by loading 10 µl of semen specimen onto a pre-warmed Makler chamber and submitted to evaluation. The values of total, progressive and rapid motility were recorded and expressed in percentages. Additional velocity parameters including Average Path Velocity (VAP, µm/s), Straight Linear Velocity (VSL, µm/s), Curvilinear Velocity (VCL, µm/s), Amplitude of Lateral Head displacement, (ALH, µm), Beat Cross Frequency (BCF, Hz), Linearity (LIN, [VSL/VCL] × 100) and Straightness (STR, [VSL/VAP] × 100), were determined. In all the trials, 8 randomly-selected microscopic fields were analyzed for each sample.

2.3. Assessment of sperm plasma membrane integrity

The sperm plasma membrane integrity was determined using the hypo-osmotic swelling assay (HOS) according with the study of Nie and Wenzel, (2001). In detail, the HOS solution was settled to ~ 100 mOsm/kg and contained 1.712 g of sucrose dissolved in 50 ml of sterile deionized water. Semen sample - 10 µl - was mixed with 100 µl pre-

warmed (37 °C) HOS solution and incubated at 37 °C for 60 min. In all six trials, for the evaluation of each sample, two hundred spermatozoa were counted and the percentage of cells with curled tails (swollen i.e intact plasma membrane) were recorded as HOS positive.

2.4. Assessment of sperm DNA integrity using SCSA

The DNA integrity of spermatozoa was assessed by sperm chromatin structure assay (SCSA), that utilizes the metachromatic properties of acridine orange (AO, Sigma-Aldrich, USA) to distinguish between denatured and native DNA in sperm, according with the procedure reported in the study of Evenson and Jost, (2000). Semen was thawed at 37 °C for 60 s and washed once using PBS solution by centrifugation at 500 *g* for 10 min. Aliquots of the thawed semen were diluted to a final concentration of 2 x 10^6 sperm/mL with TNE buffer (0.01 M Tris-Cl, 0.15 M NaCl, 1 mM EDTA, disodium pH 7.4). Then, 400 µl of acid detergent solution (0.08 N HCl, 0.15 M NaCl, 0.1% (w/v) Triton X-100, pH 1.2) was added. After 30 s, 1200 µl of AO staining solution containing 6 µg AO (2% in H_2O,) per ml staining buffer (0.037 M citric acid, 0.126 M Na_2HPO_4, 1.1 mM EDTA disodium, 0.15 M NaCl, pH 6.0), was added. Flow cytometric evaluation was conducted for n=4 trials (3^{rd}, 4^{th}, 5^{th} and 6^{th}).

2.5. Assessment of sperm mitochondrial activity

The sperm mitochondrial status was assessed using JC-10 (lipophilic cation). According to the manufacturer (JC-10 Assay for Flow Cytometry, Sigma-Aldrich, USA), JC-10 changes reversibly its fluorescence from green (monomeric status) to orange (multimeric status) when mitochondrial membrane potential is high. Frozen straws were thawed at 37 °C for 60 s.and the sperm suspension was collected into polypropylene tubes at a final concentration of 1 x 10^6 sperm/ml. One group of semen was induced for apoptosis using carbonyl cyanide m-chlorophenyl hydrazine (CCCP) 1 mM and incubated at 37 °C for 15 min and served as positive control. All groups were washed in 1 ml PBS by centrifugation at 600 *g* for 10 min, then resuspended in 500 µl of JC-10 (200x JC-10 in DMSO) and incubated 1 h at 37 °C, after that samples were centrifuged and diluted in 1 ml PBS. Flow cytometric evaluation was conducted for n=4 trials (3^{rd}, 4^{th}, 5^{th} and 6^{th}).

2.6. Evaluation of sperm for apoptosis (Annexin-V/PI-binding assay)

Translocation of phosphatidylserine (PS) phospholipids and sperm plasma membrane integrity was detected using Alexa Fluor 488 Annexin-V Apoptosis Kit (V13245, Thermo Fisher Scientific, Waltham, MA, USA) and Propidium Iodide (PI) according to Anzar et al., (2002) with some modification. Semen was thawed at 37 °C for 60 s, and washed once using PBS by centrifugation at 500 *g* for 10 min. Aliquots of semen were diluted in Annexin-V-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) to a final concentration of 1 x 10^6 spermatozoa/ml. Aliquots of diluted semen (100 µl) from each group were transferred to a 5 ml culture tubes and supplemented with 5 µl of

Annexin-V and 1 µl of PI (100 µg/ml). The tubes were gently mixed and incubated for 15 min at room temperature in the dark. Additional 400 µl of Annexin-V-binding buffer was added to each tube prior to flow cytometric evaluation. Flow cytometric evaluation was conducted immediately after the end of the staining procedure. The analyses were conducted for n=4 trials (3^{rd}, 4^{th}, 5^{th} and 6^{th}).

2.7. Flow cytometric analysis

Samples were analyzed by a FacsStar Plus flow cytometer (Becton Dickinson Immunochemistry, San Jose, CA, USA), equipped with standard optics and an air-cooled argon laser operated at 488 nm excitation and 15 mW.

In SCSA assay, after passing a 560 nm short pass dichroic mirror, the green fluorescence (FL1) was evidenced through a 515-545 nm band pass filter. The red fluorescence (FL3) was evidenced after passing a 640 nm long pass filter followed by a 650 nm long pass filter. The sheath/sample was set on ‘‘low’’, adjusted to a flow rate of 200 events/s when analyzing a sample with a concentration of 1 x 10^6 sperm/ml. Immediately after the addition of the AO staining solution, the sample was placed in the flow cytometer. Recording of the red and green fluorescence was started exactly 3 min after the beginning of the staining procedure. In each sample 10 x 10^3 cells were collected using the Cellquest software (Becton Dickinson Immunochemistry, San Jose, CA, USA). The X-mean (red) and Y-mean (green) values of each sample were recorded.

In Annexin-V/PI-binding assay: for each cell, forward light scatter (FSC), orthogonal light scatter (SSC), FITC fluorescence (FL1) and PI fluorescence (FL3) were evaluated using the Cellquest software. Acquisition gate applied in the FSC/SSC two-dimensional histogram was used to restrict the analysis to spermatozoa and to eliminate small debris and other particles for further analysis. For the gated sperm cells, four different kinds of sperm were observed. The percentages of viable spermatozoa (Annexin-V^-, PI^-), necrotic sperm (annexin-V^-, PI^+), apoptotic sperm (annexin-V^+, PI^+) and early apoptotic (annexin-V^+, PI^-) were evaluated, based on regions determined from single-stained and unstained control samples.

In mitochondrial activity assessment by JC-10: a total of 10.000 gated events/s were analyzed per sample. The sample was adjusted to a flow rate of 200 events/set. A 488 nm filter was used for excitation of JC-10. Emission filters of 535 nm and 595 nm were used to quantify the population of spermatozoa with green (JC-10 monomers) and orange (JC-10 aggregates) fluorescence, respectively. Frequency plots were prepared for FL1 (green) and FL2 (orange) to determine the percentage of the population stained green and orange. Percentage of orange stained cells was recorded, being considered as a population of cells with High Mitochondrial Membrane Potential (HMMP).

2.8. Statistical analysis

Data were analyzed using the Generalized Linear Model (GLM) procedure for repeated measurements (SPSS, Ver. 16), and presented as mean ± SEM. Pearson

correlations have been used to find the correlations between the different experimental parameters. Values with $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Effect of relaxin on motility parameters of frozen-thawed stallion semen

The mean values of sperm motility parameters evaluated by CASA analysis are presented in Table 1. Relaxin incorporation at different concentrations in extender medium during cryopreservation improved the motility parameters of frozen-thawed stallion sperm. In detail, relaxin significantly improved the total sperm motility after thawing at any examined times of incubation and the highest values were recorded with semen samples treated with 12.5 ng/ml relaxin after 90 min. post thawing, or at 25 ng/ml relaxin concentration after 30 and 60 min. post thawing (up to $P<0.001$; Table 1). Whereas, samples treated with 50 ng/ml relaxin showed the highest value after 120 min. post thawing ($P<0.05$; Table 1). Similarly, the progressive motility was significantly improved in relaxin-treated samples immediately after thawing and after 30, 60, 90 and 120 min. of incubation and the highest values were recorded when 12.5 ng/ml relaxin was added ($P<0.05$; Table 1). Regarding the rapid motility, it was significantly improved by relaxin addition with highest values obtained at the same concentrations and times identified for progressive motility (up to $P<0.001$; Table 1).

3.2. Effect of relaxin on velocity parameters of frozen-thawed stallion semen

The effects of relaxin on sperm velocity parameters are detailed in Table 2. Relaxin incorporation at different concentrations in extender medium during cryopreservation significantly improved several velocity parameters of stallion semen after thawing and incubation for 0, 30, 60 and 120 min. at 37 °C. In detail, VAP, VSL and VCL, velocity parameters also associated with a capacitated state of spermatozoa, were significantly improved by relaxin addition especially at 12.5 and 25 ng/ml from 0 up to 120 min. of incubation (up to $P<0.001$; Table 2). As well as, STR and LIN, which provide important information about the linearity of the sperm velocity path, were significantly improved after thawing by relaxin addition and highest values were recorded especially at 12.5 ng/ml from 0 up to 120 min. of incubation (up to $P<0.001$; Table 2). Similarly, ALH and BCF, other velocity parameters correlated with sperm head movement, were also affected by relaxin addition.

3.3. Effect of relaxin on plasma membrane and DNA integrity and mitochondrial membrane potential

The effects of relaxin on sperm vitality are detailed in Table 3. On the basis of the results obtained on sperm motility, the timing point of 60 min. was selected as the one in which best results were obtained, thus it was used for further analyze the effect of relaxin on other sperm quality parameters. Interestingly, relaxin at all tested concentrations,

significantly improved the sperm mitochondrial membrane potential (HMMP) compared to the control group ($P<0.01$). No statistical differences were found on the effects of relaxin on plasma membrane and DNA integrities at any tested concentrations (Table 3).

3.4. Effect of relaxin on semen apoptosis

The effects of relaxin on sperm apoptosis are presented in Table 4. At all tested concentrations, relaxin significantly ($P<0.02$) decreased the percentage of apoptotic cells. Higher values of normal viable sperm were found in relaxin-treated samples at 12.5, 50 and 100 ng/ml, even if there were not significant differences (Table 4). Whereas, no statistical differences were found between the percentage of normal viable and necrotic sperm cells compared with controls.

3.5. Correlations between the different sperm quality parameters

The correlations between the different semen quality parameters, calculated on the basis of the effects of relaxin, are summarized in Table 5. The total sperm motility has a significant positive correlation with progressive motility, rapid motility, intact DNA ($P<0.01$) and HMMP ($P<0.05$), whereas it is negatively correlated with apoptotic sperm, VAP ($P<0.05$), VCL, VSL, ALH, STR and LIN ($P<0.01$). The progressive motility is positively correlated with rapid motility ($P<0.01$) and HMMP ($P<0.05$), whereas it is negatively associated with VAP, ALH, BCF and STR ($P<0.01$).

4. Discussion

Semen cryopreservation plays an important role in preserving genetic materials in humans and domestic animals (Axner et al., 2004). However, the cryopreservation process induces detrimental structural effects on spermatozoa during freezing and thawing procedures, as a result of exposure to different stressful factors including thermal, chemical, osmotic, mechanical and oxidative stress (Holt et al., 1992). These changes result in perturbations to the sperm organelles, changes in membrane fluidity and enzymatic viability, loss of plasma membrane and acrosome integrity and finally decreased sperm motility (Alvarez and Storey, 1983; Woelders et al., 1997). All these alterations contribute to decrease the semen fertilizing capability (Tekin et al., 2006) and in particular, the equine sperm appear to be extremely sensitive to alterations generated by the cryopreservation process (Ball, 2008; Gibb and Aitken, 2016). However, differences exist in the ability of sperm to survive cryopreservation, also between individual males within a species. Thus, with the aim to eliminate the potential individual stallion variability, the semen samples from all three stallions were pooled together, as it was also reported in previous studies in the same species (Seifi-Jamadi et al., 2016; Shojaeian et al., 2018; Nouri et al., 2018).

In different species, relaxin has been demonstrated to improve sperm motility (Ferlin et al., 2012, Miah et al., 2007, Miah et al., 2008; Feugang et al., 2015), capacitation and acrosome reaction (Miah et al., 2008; Miah et al., 2011), and fertilizing ability of spermatozoa (Han et al., 2006; Elkhawagah et al., 2015). However, in previous studies, the

effects of relaxin have been investigated on fresh semen (Han et al., 2006) or on cryopreserved semen with this compound added during thawing procedures (reviewed by Miah et al., 2015). Instead, in the present study, by using a different methodological approach we improved several sperm quality parameters of cryopreserved stallion sperm by the incorporation of relaxin at different concentrations (12.5, 25, 50 and 100 ng/ml) in the extender medium before the cryopreservation process.

Sperm motility, evaluated by CASA analysis, is one of the most reliable parameter associated with sperm fertilizing potential (Verstegen et al., 2002). In addition, Voss et al., (1981) stated that spermatozoa motility is the most reliable method to estimates field fertility. Our results revealed that 12.5 and 25 ng/ml relaxin significantly improved the total and the progressive sperm motility after thawing at different incubation times. Moreover, other sperm velocity parameters were also improved by relaxin addition, and these parameters such as VCL, VSL and ALH positively correlate with sperm capacitation and fertility. However, semen samples did not show excellent post-thawing motility and it might depend from both the use of sperm collected and frozen at the end of the commercial season and the use of nitrogen vapors instead of programmable freezers for cryopreservation procedure. The improvement of the semen quality, even with these conditions, strengthens the consideration on the positive effects of the relaxin addition.

Our result are in agreement with other studies performed in different species. In detail, relaxin has been found to improve sperm motility of boar (Feugang et al., 2015; Miah et al., 2006; Kohsaka et al., 2001), bovine (Miah et al., 2007) and buffalo (Elkhawagah et al., 2015) spermatozoa. The influence of relaxin on sperm motility and fertility has been suggested to occur through specific cell-surface receptors on sperm head and tail (Kohsaka et al., 2003; Feugang et al., 2015). Our result in the equine species could be compared with the study reported by Burns and Fleming, (1989), in which a significant improvement in the total sperm motility of frozen-thawed stallion semen treated with 400 ng/ml relaxin for 1 hr at room temperature, was identified. However, also in this study, relaxin was supplemented in the sperm-thawing medium.

It has been stated that the relationship between motility and fertility of stallion frozen semen is not the only measure of the fertilizing potential (Bataille et al., 1990). In fact, Graham, (1996) and Katila, (2001) recommended that spermatozoa should possess several quality parameters including motility, normal morphology, plasma membrane integrity (Andrabi et al., 2016; Aurich, 2005; Baumber et al., 2005) sufficient metabolism for energy production and membrane integrity to acquire the fertilizing ability. Therefore, HOS test was performed to test the effect of relaxin on sperm quality parameters of stallion spermatozoa. However, HOS test did not reveal any difference within the experimental groups in accordance with that reported by Feugang et al., (2015) who denied the relationship between relaxin treatment and boar sperm plasma membrane integrity.

By the evaluation of the effects of relaxin on sperm DNA integrity by using SCSA, our results did not show any differences within experimental groups. It has been stated that the loss of sperm fertilizing ability after freezing/thawing in boar could be attributed to factors other than sperm chromatin structural damage, as it is very resistant (Evenson et al., 1994). However, Neild et al., (2003) and Ortega-Ferrusola et al., (2009) stated that apoptosis-like mechanisms and lipid peroxidation of plasma membrane are associated with cryopreserved equine sperm premature aging and DNA fragmentation.

Semen cryopreservation interferes with sperm-membrane functions (Chaveiro et al., 2007) inducing membrane phospholipids asymmetry and progressively damaging the cellular integrity (Martin et al., 1995) with the induction of apoptotic-like changes (Crabo, 2001). Our results showed that all concentrations of relaxin significantly decreased the percentage of apoptotic spermatozoa compared to the control group, and the lowest value was recorded with relaxin 100 ng/ml. These results are in agreement with that reported by Ferlin et al., (2012) who found that treatment of human sperm with 100 nM relaxin prevent apoptosis and increased the percentage of viable sperm.

Sperm mitochondria are considered to be the site for production of the adenosine-triphosphate (ATP), which is essential for sperm motility (Silva and Gadella, 2006; Amaral et al., 2013). Therefore, the sperm mitochondrial status is an important factor for sperm fertilizing ability. Osmotic shock is a major factor in sperm damage during cryopreservation (Prien and Iacovides, 2016) that leads to a loss in viability by decreasing the mitochondrial membrane potential (Papa et al., 2011). In our study, we improved the mitochondrial membrane potential of cryopreserved stallion semen by the incorporation of relaxin in the freezing medium at different concentrations. This is in agreement with that reported by Ferlin et al., (2012) who found that relaxin at 10 and 100 nM preserved HMMP of human sperm. Moreover, we found a significant positive correlation between motility and quality parameters such as mitochondrial membrane potential and sperm apoptosis.

These results reveal that relaxin could be added in the extender medium, before the sperm cryopreservation procedures rather than in the post-thawing media or even in the in vitro fertilization/embryo culture media. Our methodological approach could be more suitable for in field applications, avoiding any type of treatment of the semen after thawing procedures and/or before the artificial insemination and we can recommend the use also in reduced quality sperm ejaculate

5. Conclusions

In the present study we improved the post-thawing fertility parameters of cryopreserved stallion semen by incorporation of relaxin at different concentrations (12.5, 25, 50 and 100 ng/ml) in extender medium, before cryopreservation procedures. Our results revealed that 12.5 and 25 ng/ml relaxin had a positive stimulatory effect on different quality parameters of frozen-thawed semen including an improvement of sperm motility and velocity parameters in addition to an increase of the percentage of sperm with higher mitochondrial membrane potential and a reduction of sperm apoptosis.

Conflict of interest

No conflict of interest was reported by the authors.

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